Functional analysis of the miRNA-mRNA interaction network in *C. elegans*

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MicroRNAs (miRNAs) are conserved small non-coding RNAs that typically regulate gene expression by binding to the 3' untranslated region (UTR) of mRNAs. Developmental functions of miRNAs have been extensively studied, but additional roles in various cellular processes remain to be understood. The investigation of the biological importance of individual miRNA-target interactions and the miRNA-target interaction network as a whole has been an exciting and challenging field of study. Here we briefly discuss the contributions our lab has made to our understanding of the physiological impact of this miRNA-network in *C. elegans*, in the context of recent studies in this advancing field. These studies have advanced our knowledge of the role of miRNAs in ensuring a robust cellular response to different physiological conditions. We briefly outline the genetic, biochemical, and computational strategies utilized to understand miRNA functions and discuss our recent study of the miRNA-interaction network in neurons and potential directions for future studies.

Hundreds of microRNAs (miRNAs) interact with thousands of target mRNAs to maintain proper gene expression patterns under various physiological functions. The complexity of the miRNA-target interaction network presents a great challenge for scientists to dissect the roles for specific miRNAs or miRNA-target interactions in specific biological processes.¹⁻⁵ Furthermore, miRNA regulation is only

one of the multiple mechanisms involved in regulating gene expression dynamics. Mechanisms such as transcriptional repression and post-translational degradation or modification may also act semi-redundantly with miRNA-mediated gene silencing. Therefore, miRNAs may mostly function to enhance the robustness of dynamic changes in gene expression under different physiological conditions.^{6,7} This hypothesis is consistent with accumulated data indicating that individual miRNA-target interactions rarely play pivotal roles in regulating development or other physiological functions under favorable growth conditions.²⁻⁴ To identify regulatory miRNA-target interactions and their physiological importance under different conditions, we and others have employed new biochemical and genetic approaches.

Computational predictions can allow for the identification of potential microRNA targets.⁸ However, discerning bona fide targets under real physiological conditions, spatially and temporally, is necessary for understanding miRNA biology. After our lab's initial discovery of the critical role of GW182 family proteins (AIN-1 and AIN-2) in miRNA-induced silencing complexes (miRISCs), we developed an immunoprecipitation-based biochemical method to systematically address this issue in *C. elegans*.^{7,9-11} After immunoprecipitating cross-linked, GFP-tagged AIN-1 or AIN-2 (which associates with miRISC complexes) with an anti-GFP antibody (AIN-IP), RNA is subsequently extracted from the miRISC for microarray analysis (mRNAs) and

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cloned for deep-sequencing analysis (miRNAs). These AIN-IP experiments were successful in systematically isolating and identifying thousands of mRNAs and hundreds of miRNAs associated with miRISC, which is mostly consistent with the results from analyses by others using another miRISC immunoprecipitation method.¹² Applying bioinformatics analysis on the AIN-1/2-associated mRNAs from the initial study by Zhang et al. also led to the development of a new target prediction program by Dr. Molly Hammell in the Ambros lab.^{10,13} This methodology gave us a broad perspective of the types of mRNAs, their possible functions, and their potential miRNAs regulators—providing us with insight into the miRNA-target interaction network. To further increase the sensitivity of this methodology, our lab collaborated with the Ambros lab to isolate and analyze miRISCs at specific developmental stages and revealed that, throughout development, miRNAs seem to target genes involved in signaling and regulatory functions over those involved in housekeeping.¹¹ This approach was further developed into analyzing miRISCs in specific tissues including the gut, muscles, and neurons.^{7,21} For example, we found that among more than 500 mRNAs associated with intestinal miRISCs, about one quarter are known pathogen-responsive genes.⁷ These studies show that biochemical isolation and analysis of miRNAs and mRNAs can provide insight into potential functions of miRNAs, complementing those provided by computational predictions and analyses.

In addition to biochemical approaches, genetic approaches may also be applied to uncover miRNA function. Antagonizing miRISC function through mutations in individual GW182 (*ain-1* and *ain-2*) or Argonaut (*alg-1* and *alg-2*) genes systematically compromises total miRNA function. This methodology is often favorable, because it helps bypass the inherent redundancy between miRNA family members and permits us to interrogate and identify potential functions of miRNAs by looking at defects under various conditions.^{14–17} For example, our initial finding that *ain-1(lf)* mutants

were sensitive to L1 starvation indicated that miRNAs play an important role in regulating L1 starvation survival.¹⁷ This led to the identification of a prominent role of miR-71's regulatory interaction with multiple target genes including several acting in the Insulin/IGF-1 signaling (IIS) pathway for starvation survival. These results are consistent with the analysis of miR-71 by others, including miR-71's association in longevity via IIS.^{6,18,19} These studies show that miR-71 also plays an important role in regulating L1 starvation survival, in addition to longevity.

Moreover, since compromising miRISC with mutations in *ain-1* or *ain-2* creates a more sensitive genetic background, it is possible to perform more elegant genetic analyses to further identify miRNA functions. Previous experiments have shown that interrupting global miRISC functions, individual miRNAs, key regulators of development, or any of these in combination can help to further elucidate the roles of individual miRNAs.^{14,20} These studies found that loss of individual miRNAs enhanced the phenotype of *alg-1* mutants, helping to identify further functions of miRNAs. Conversely, using a sensitized background compromising individual miRNAs (*let-7*) can help identify other potential interactors.²⁰ For example, Brenner et al. found that, while *mir-59(lf)* mutants look superficially wild-type, *mir-59(lf)* increased adult lethality within an *alg-1* mutant background, suggesting that miR-59 may have more important functions, which they suggested was in gonad migration.¹⁴ These studies using sensitized backgrounds are important because they allow for identification of novel functions of miRNAs that would otherwise be missed.

Although biochemical or genetic approaches can be used to elucidate the physiological functions of miRNAs, complementing one approach with the other can lead to an enhanced understanding of miRNA biology. As demonstrated by Kudlow et al. from our lab, complex biochemical and statistical analyses can be used to uncover a miRNA-target interaction network in a specific tissue (gut) for a specific function (pathogen response).⁷

This paper showed that about 17 miRNAs regulate more than 100 pathogen-inducible target mRNAs specifically in the gut. When complemented by genetics, this work discovered that *ain-1(lf)* mutants had favorable survivability during a pathogenic insult and found several individual miRNAs that, when mutated, led to modest increases in survival. This work indicates that one major miRNA activity in the intestine is devoted to prevent high-level expression of pathogen-responsive genes that, while beneficial in fighting against pathogen attack, are toxic to the animals' health in the absence of pathogen. This study demonstrated well why this approach is effective in uncovering functions of miRNAs-targets interaction networks and provoked our thinking regarding the fundamental functions of most miRNA-target interactions.

Our most recent study used a combination of the techniques discussed above to elucidate a function of miRNAs in neurons. We used an *unc-3(lf)* mutant to create a genetically sensitized background to further investigate functions of miRNAs in neurons.²¹ UNC-3 is a transcription factor responsible for neuronal development in *C. elegans*, and compromising UNC-3 function interrupts motor neuronal development and affects ASI chemosensory neurons. Overall, *unc-3(lf)* mutants had defects in the nervous system and we reasoned that if we could compromise these genetic networks in a neuronal tissue, we could use this as an “enhancer” to identify miRNA functions within this specific tissue. This thought was supported by our finding that compromising the UNC-3 transcription factor along with compromising AIN-1 resulted in aberrant dauer formation. This increase in dauer formation is an enhancement of the *unc-3(lf)* mutant's ASI differentiation defect, and not the motor neuron defect. Interestingly, we found that this effect was specific for *ain-1*, and not for *ain-2* or *alg-2*, which suggests that there is differential expression of miRISC components across different tissues. Using this physiological function to justify an in depth search into the neuronal miRNA-target interaction network, we performed a neuronal-restricted AIN-IP, expecting to find a

robust enrichment of dauer regulators, in a similar manner to the enrichment of pathogen-responsive genes in the intestine in our previous study.⁷ We were able to identify individual miRNAs involved in dauer formation and showed several important regulators of dauer formation enriched in neuronal miRISC. We identified several lesser known members of the let-7 family that seem to be exclusively expressed in neurons; the importance of this finding requires more investigation. Additionally, we show differential roles of the miR-58 family members (miR-58, -80, -81, -82). miR-58, -80, and -82 have been shown to be required for dauer formation, while we show that miR-81 represses dauer formation.²² Because these miRNAs share a similar seed site, the difference in function may be due to differences in spatial or temporal expression. These results highlight the importance of identifying spatial and temporal expression of miRNAs in understanding miRNA biology.

Although some information was gleaned from the neuronal AIN-IP that was performed on all neurons of asynchronous stages, there is still much to understand about the neuronal miRNA-target network in *C. elegans*. Consequently, the heterogeneity of enriched mRNAs in neuronal miRISCs may be caused by the differing patterns of gene expression and varying functions of individual neurons and at different stages. One potential way to resolve this issue is to use individual cell-based AIN-IPs, as opposed to all neurons or developmentally synchronous AIN-IPs vs. asynchronous populations. This would allow for miRNA-target identification within a cell type or developmental stage, allowing for increased sensitivity to detect weakly expressed, cell-specific, or stage-specific transcripts. Moreover, we found that a significantly greater percentage of mRNAs are associated with miRISCs in the neuron than in other major tissues.²¹ This may suggest that a much greater portion of neuronal transcripts, including many located in the axon away from the cell body, are miRNA regulated. The significance of this observation remains to be understood. Additionally, although we found many regulators of dauer formation enriched

in our neuronal IP, direct miRNA-target interactions were not readily apparent. Instead, it would be interesting to understand the cellular mechanisms by which miRNAs are readily integrated into signaling pathways in neurons to maintain organismal stress responses to environmental insults. One approach to understanding this phenomenon would be to apply the neuronal AIN-IP to *C. elegans* exposed to various stress conditions or induced dauer larvae. These approaches can help identify miRNA-target networks that are induced or suppressed during environmental stress, which may provide insights into potential networks that collaborate with miRNAs. Lastly, our observation that *ain-1(lf)* enhanced the *unc-3(lf)* dauer formation phenotype suggests that identifying other potential genetic interactions between *ain-1*, *ain-2*, *alg-1*, or *alg-2* mutations (in specific tissues or in general) with other regulatory factors may be effective for further discovery and understanding of miRNA functions and how they collaborate with other cellular machinery to regulate cellular and organismal physiology.

Studies into understanding miRNA function from our lab and others have taken various approaches which have provided varying levels of insight into physiological functions of miRNAs. We believe that a combination of systematic genetics, biochemistry, and statistics is an effective way of elucidating miRNA function. Our most recent study provides an example of genetically impacting a specific tissue (through an *unc-3* mutant) to understand miRNA functions. Similar approaches are expected in our lab and other labs to identify cellular functions, especially stress responses, of which the miRNA-target interaction network collaborates with other regulatory systems (transcription, protein degradation, etc.) to warrant the robustness of gene expression dynamics.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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